THE LANCET

Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Fitzgerald K, Frank-Kamenetsky M, Shulga-Morskaya S, et al. Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. *Lancet* 2013; published online Oct 3. http://dx.doi.org/10.1016/S0140-6736(13)61914-5.

Supplemental Table 1: Phase 1: percentage change from baseline for lipid parameters, by dose group

	0·015 mg/kg (n=3)	0·045 mg/kg (n=3)	0·090 mg/kg (n=3)	0·150 mg/kg (n=3)	0·250 mg/kg (n=6)	0·400 mg/kg (n=6)	Placebo (n=8)
Fasting serum total cholesterol							
Mean percentage change from baseline[1]	-6.6%	-5.4%	-16.4%	-17.7%	-20.7%	-23.5%	-10.8%
Time to group nadir (days)[2]	3	3	14	3	3	14	3
p value *vs* baseline[3]	0.1430	0.1376	0.0255	0.0098	0.0005	0.0020	0.0241
Mean percentage change from baseline relative to placebo[4]	-0.7%	-5.3%	-17.8%	-15.2%	-17.5%	-26.9%	
Time to group nadir relative to placebo (days)[5]	1	14	14	14	14	10	
p value relative to placebo[4]	0.9092	0.4110	0.0031*	0.0128	0.0003*	<.0001*	
Mean percentage change *vs* placebo[6]	-1.0%	-6.3%	-18.9%	-16.2%	-18.6%	-30.4%	
Time to group nadir *vs* placebo (days)[7]	1	14	14	14	14	10	
p value *vs* placebo[6]	0.8741	0.3106	0.0023*	0.0087	0.0002*	<.0001*	

	0·015 mg/kg (n=3)	0·045 mg/kg (n=3)	0·090 mg/kg (n=3)	0·150 mg/kg (n=3)	0·250 mg/kg (n=6)	0·400 mg/kg (n=6)	Placebo (n=8)
Fasting serum HDL cholesterol							
Mean percentage change from baseline[1]	-10.5%	-2.0%	-16.9%	-19.6%	-21.0%	-19.5%	-13.1%
Time to group nadir (days)[2]	3	2	4	4	3	3	3
p value *vs* baseline[3]	0.2822	0.7167	0.1070	0.0337	0.0078	0.0040	0.0231
Mean percentage change from baseline relative to placebo[4]	-2.4%	0.1%	-9.0%	-8.2%	-8.6%	-6.7%	
Time to group nadir relative to placebo (days)[5]	2	28	28	14	3	3	
p value relative to placebo[4]	0.7328	0.9883	0.1823	0.2379	0.1103	0.2162	
Mean percentage change *vs* placebo[6]	-2.3%	0.1%	-9.7%	-7.6%	-7.5%	-6.3%	
Time to group nadir *vs* placebo (days)[7]	1	28	28	14	3	1	
p value *vs* placebo[6]	0.7339	0.9845	0.1545	0.2729	0.1679	0.2507	

	0·015 mg/kg (n=3)	0·045 mg/kg (n=3)	0·090 mg/kg (n=3)	0·150 mg/kg (n=3)	0·250 mg/kg (n=6)	0·400 mg/kg (n=6)	Placebo (n=8)
Fasting triglycerides							
Mean percentage change from baseline[1]	47.8%	86.8%	66.7%	52.6%	37.5%	42.8%	43.5%
Time to group nadir (days)[2]	1	28	1	1	1	1	28
p value *vs* baseline[3]	0.4260	0.0448	0.0945	0.0666	0.0156	0.0058	0.0833
Mean percentage change from baseline relative to placebo[4]	-11.6%	-34.7%	-12.6%	-8.9%	-17.0%	-28.7%	
Time to group nadir relative to placebo (days)[5]	4	4	7	1	1	10	
p value relative to placebo[4]	0.5868	0.2288	0.5530	0.6837	0.3067	0.2130	
Mean percentage change *vs* placebo[6]	-20.6%	-131.0%	-16.8%	-24.5%	-37.0%	-91.7%	
Time to group nadir *vs* placebo (days)[7]	4	4	7	1	1	4	
p value *vs* placebo[6]	0.7307	0.1611	0.7789	0.6824	0.4394	0.0568	

	0·015 mg/kg (n=3)	0·045 mg/kg (n=3)	0·090 mg/kg (n=3)	0·150 mg/kg (n=3)	0·250 mg/kg (n=6)	0·400 mg/kg (n=6)	Placebo (n=8)
Fasting VLDL cholesterol							
Mean percentage change from baseline[1]	-2.8%	-44.8%	-33.3%	4.3%	-13.4%	-27.3%	-36.2%
Time to group nadir (days)[2]	1	14	1	1	1	14	1
p value *vs* baseline[3]	0.9254	0.5207	0.2122	0.1841	0.4924	0.2355	0.0018
Mean percentage change from baseline relative to placebo[4]	7.4%	-37.7%	4.0%	29.0%	5.2%	-21.2%	
Time to group nadir relative to placebo (days)[5]	3	14	1	28	7	10	
p value relative to placebo[4]	0.8101	0.1090	0.8938	0.3930	0.8311	0.4961	
Mean percentage change *vs* placebo[6]	1.7%	-8.5%	-0.6%	14.4%	2.1%	-22.6%	
Time to group nadir *vs* placebo (days)[7]	3	14	7	28	21	28	
p value *vs* placebo[6]	0.9643	0.8222	0.9865	0.7058	0.9453	0.4584	

	0·015 mg/kg (n=3)	0·045 mg/kg (n=3)	0·090 mg/kg (n=3)	0·150 mg/kg (n=3)	0·250 mg/kg (n=6)	0·400 mg/kg (n=6)	Placebo (n=8)
Fasting lipoprotein A							
Mean percentage change from baseline[1]	nd	nd	nd	-30.6%	-19.0%	-17.6%	-14.5%
Time to group nadir (days)[2]				4	14	14	21
p value *vs* baseline[3]				0.1928	0.0009	0.0430	0.6064
Mean percentage change from baseline relative to placebo[4]	nd	nd	nd	-25.5%	-14.1%	-11.1%	
Time to group nadir relative to placebo (days)[5]				4	14	14	
p value relative to placebo[4]				0.0854	0.2726	0.4079	
Mean percentage change *vs* placebo[6]	nd	nd	nd	-23.3%	-17.7%	-14.4%	
Time to group nadir *vs* placebo (days)[7]				4	14	14	
p value *vs* placebo[6]				0.1068	0.1319	0.2332	

	0·015 mg/kg (n=3)	0·045 mg/kg (n=3)	0·090 mg/kg (n=3)	0·150 mg/kg (n=3)	0·250 mg/kg (n=6)	0·400 mg/kg (n=6)	Placebo (n=8)
Fasting apolipoprotein B							
Mean percentage change from baseline[1]	nd	nd	nd	-22.1%	-15.9%	-21.9%	-8.1%
Time to group nadir (days)[2]				4	4	14	4
p value *vs* baseline[3]				0.0738	0.0239	0.0055	0.0855
Mean percentage change from baseline relative to placebo[4]	nd	nd	nd	-18.8%	-18.1%	-26.2%	
Time to group nadir relative to placebo (days)[5]				14	14	14	
p value relative to placebo[4]				0.0463	0.0197	0.0006	
Mean percentage change *vs* placebo[6]	nd	nd	nd	-20.0%	-20.1%	-28.4%	
Time to group nadir *vs* placebo (days)[7]				14	14	14	
p value *vs* placebo[6]				0.0393	0.0112	0.0005	

nd = not done.

Note: Samples for lipoprotein A and apolipoprotein B were only collected for participants in the 150, 250, and 400 µg/kg dose groups.

[1] Nadir for percentage change from baseline is derived from the logs of the fold change from baseline for each dose group at each timepoint. Fold change from baseline is defined as the ratio of each subject's post-baseline value to their baseline value. For this table, the minimum log of fold change value is converted back to percentage change by exp(log(fold change)-1) * 100.

[2] Study day at which the minimum mean (log) fold change occurred for each group.

[3] At nadir, p value is generated from a paired *t* test, testing log(FCHG) = 0 or log(value at nadir) = log(baseline value).

[4] Nadir for fold change normalised to placebo is derived from the estimated values of (log of active group mean fold change – log of placebo group mean fold change) generated from a repeated-measure analysis of covariance, modelling log of fold change from baseline as a function of treatment, visit, treatment-by-visit interaction, with baseline as a covariate, and subject as a random effect. p values for pairwise comparison with the placebo group are also generated from the same model.

[5] Study day at which the minimum of the difference between log of active group mean fold change and log of placebo group mean fold change occurred.

[6] Nadir for percentage change *vs* placebo is derived from the estimated values of (active group mean percentage change – placebo group mean percentage change) generated from a repeated-measure analysis of covariance, modelling percentage change from baseline as a function of treatment, visit, treatment-by-visit interaction, with baseline as a covariate, and subject as a random effect. p values for pairwise comparison with the placebo group are also generated from the same model.

[7] Study day at which the greatest negative difference between active group percentage change mean and placebo group percentage change mean occurred.

Supplemental Table 2: Phase 1: LDL cholesterol results for individual participants treated with single doses of ALN-PCS or placebo

Participant number	Dose (mg/kg)	Fasting LDL cholesterol (mmol/L)[1]									
		Baseline[2]	Day 2	Day 4	Day 7	Day 10	Day 14	Day 17	Day 21	Day 28	Day 42
001-0001	0.015	4.2	4.0	3.7	3.9	nd	3.8	nd	3.4	3.7	3.1
001-0002	Placebo	6.2	5.5	5.4	5.1	nd	5.6	nd	6.1	5.8	5.4
001-0003	0.015	4.8	3.9	4.3	4.7	nd	4.7	nd	5.0	4.1	4.5
001-0004	0.015	3.4	3.1	2.9	3.6	nd	3.6	nd	4.3	3.5	3.4
001-0005	Placebo	4.4	3.6	2.6	4.0	nd	4.6	nd	4.4	4.2	3.6
001-0006	0.045	3.1	2.4	3.2	3.4	nd	3.1	nd	2.7	2.9	3.3
001-0007	0.045	4.1	3.8	3.1	3.7	nd	3.4	nd	3.7	4.2	4.5
001-0008	0.045	3.6	3.1	nd	nd	nd	3.3	nd	4.0	4.4	4.1
001-0009	0.090	4.6	4.3	4.0	4.2	nd	3.4	nd	4.7	4.3	3.8
001-0010	Placebo	3.6	3.7	3.1	4.3	nd	4.4	nd	4.0	3.8	2.9
001-1011	0.090	3.7	2.6	2.2	3.1	nd	3.0	nd	2.5	2.6	3.4
001-0012	0.090	4.1	3.1	2.9	3.1	nd	3.3	nd	3.3	3.6	3.0
001-0013	Placebo	4.1	3.3	3.6	4.2	nd	3.6	nd	3.5	2.8	3.3
001-0014	0.150	4.0	3.4	2.5	3.5	nd	3.4	nd	3.9	3.7	3.5
001-0015	0.150	3.8	3.5	2.3	2.9	nd	2.5	nd	3.1	3.4	4.1
002-0016	0.150	3.3	2.5	2.4	2.8	nd	3.2	nd	2.5	3.0	3.0
002-0017	0.250	3.1	2.6	2.1	2.2	nd	2.5	nd	2.7	2.7	3.1
002-0018	Placebo	3.1	2.4	2.4	3.0	nd	3.4	nd	3.0	2.6	2.6
002-0019	0.250	3.5	2.8	2.3	2.9	nd	2.0	nd	3.0	2.6	3.1
002-0020	0.250	3.7	3.1	1.9	2.7	nd	2.5	nd	3.0	2.8	4.2
002-0021	0.250	3.9	3.2	3.1	3.2	nd	2.9	nd	2.5	3.4	3.5
002-0022	Placebo	3.6	2.9	2.8	3.0	nd	2.8	nd	2.9	3.5	3.1
002-0023	0.250	3.3	2.3	1.7	2.5	nd	2.8	nd	2.9	2.4	1.9
002-0024	0.250	3.9	3.0	3.0	2.9	nd	3.4	nd	3.7	3.7	3.8
002-0025	Placebo	3.1	3.1	2.4	3.5	4.2	4.0	3.9	3.7	3.9	4.4
002-0026[3]	0.400	2.9	2.6	1.9	1.7	1.6	1.6	1.6	1.7	2.1	2.5
002-0027	0.400	3.4	2.5	1.7	1.9	2.0	1.8	1.9	2.4	2.2	2.6
002-0028	0.400	3.1	2.4	1.9	2.0	2.2	2.1	2.1	2.5	2.6	3.3
002-0029	0.400	3.8	3.3	3.2	3.5	3.1	3.4	3.5	3.3	4.5	4.5
002-0030	Placebo	3.3	2.8	2.6	3.0	3.6	4.1	3.6	3.7	3.2	2.9
002-0031	0.400	3.9	3.7	3.0	2.2	2.9	2.7	2.9	2.5	3.3	3.7
002-0032	0.400	4.2	3.1	2.2	3.1	3.2	3.1	2.9	3.5	3.5	3.9

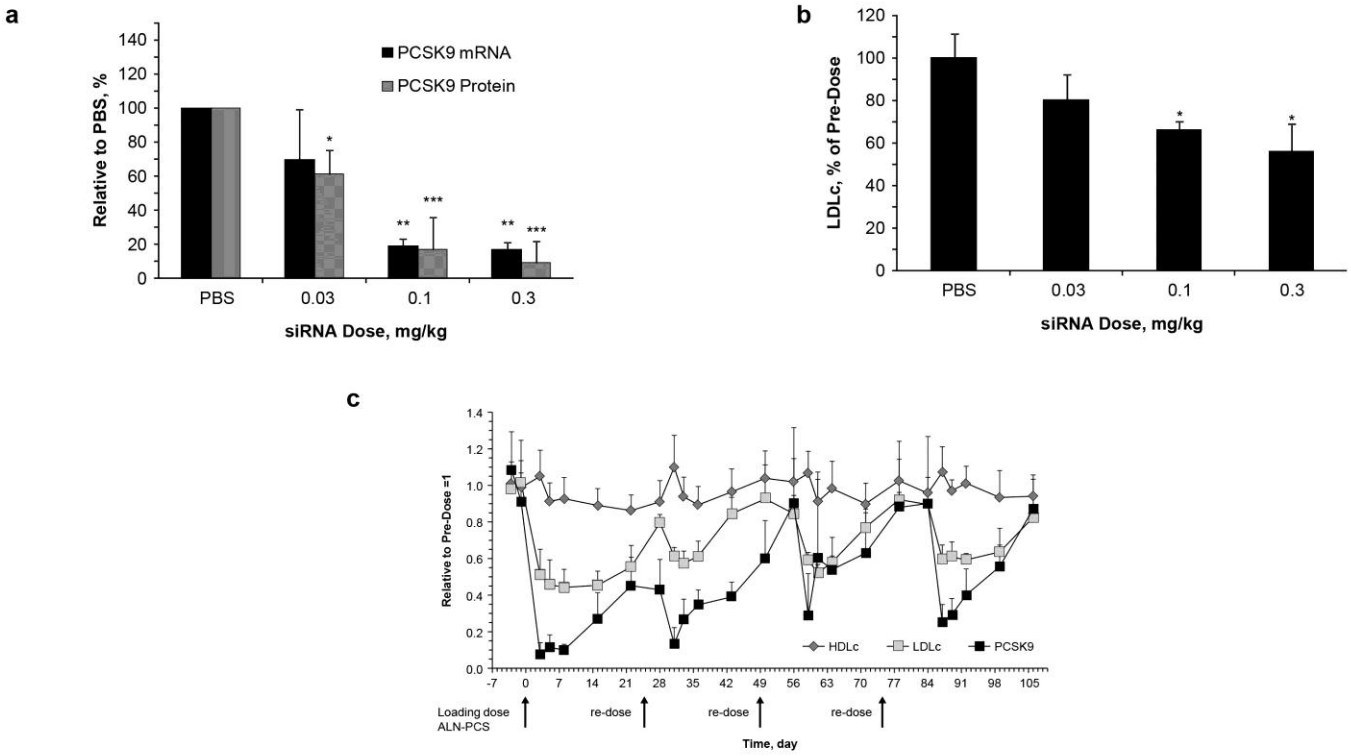
nd = not done.

[1] Based on β-quantification.

[2] Baseline averages of at least four pre-dose measurements.

[3] To be eligible for the study, at least two pre-dose LDL cholesterol measurements needed to be above 3.0 mmol/L. Patient 002-0026 had two LDL cholesterol pre-dose measurements of 3.2 mmol/L and 3.3 mmol/L.

Non-human primates — preclinical data



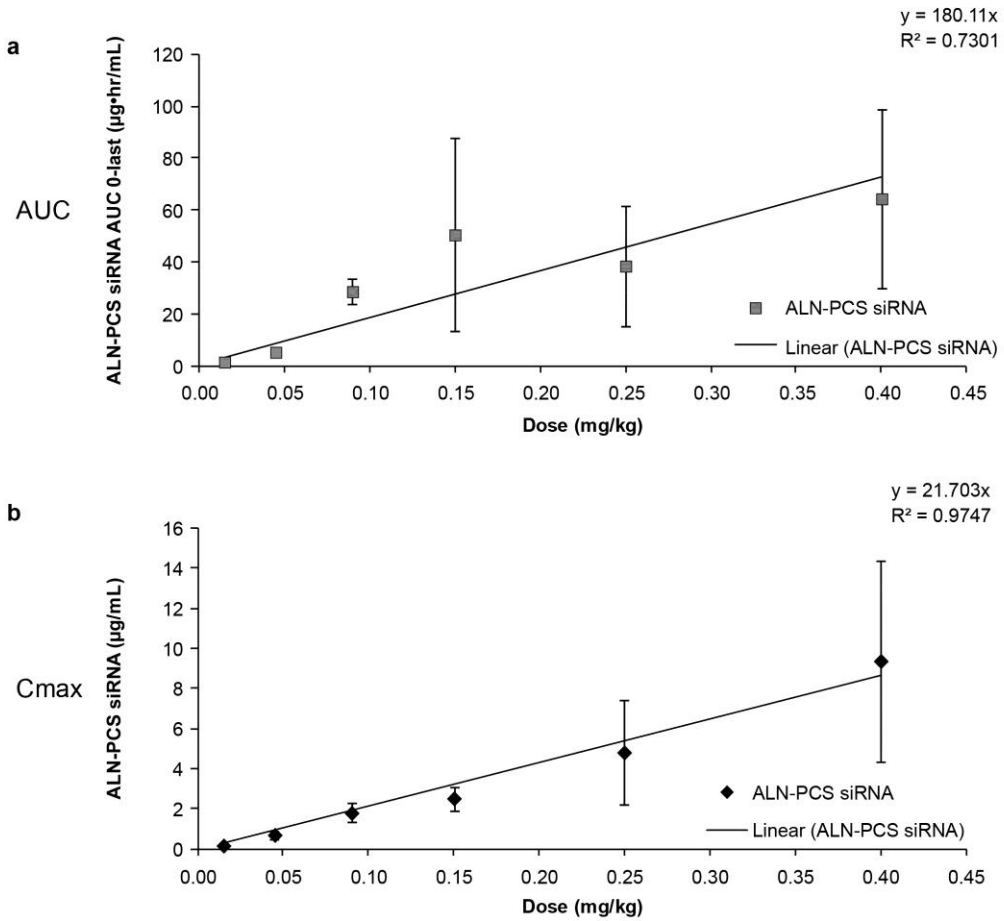
Supplemental Figure 1. ALN-PCS treatment reduces PCSK9 transcript, PCSK9 plasma protein and serum LDL-C levels in NHPs

A) Hepatic PCSK9/GAPDH mRNA and serum PCSK9 protein levels on Day 2 post-dosing with ALN-PCS or PBS. Columns represent group means ± 1 SEM, normalized to the PBS group. Asterisks indicate significance of Tukey's *post hoc* tests *vs.* control: *, P<0.05; **, P<0.01; ***, P<0.001. Values for PCSK9 protein levels (ELISA) are presented as a mean percentage of the pre-dose measurement (average of Day -1, Day -3). ANOVAs of the effect of treatment on PCSK9 mRNA and protein were both significant (F = 9.61, 9.21; P = 0.002, 0.002). The following pairwise comparisons of individual dose groups vs. control were significant according to Tukey's post hoc tests. mRNA: 0.1 mg/kg, t = -4.35, P = 0.005; 0.3 mg/kg, t = -4.446, P = 0.005. Protein: 0.1 mg/kg, t = -4.157, P = 0.007; 0.3 mg/kg, t = -4.361, P = 0.005.

B) Direct LDL-C measurements of serum on Day 2 post-dosing with ALN-PCS or PBS. Values for LDL-C concentrations are presented as a mean percentage of the pre-dose measurement (average of Day -1, Day -3). Columns represent group means ± 1 SEM, normalized to the PBS group. Asterisks indicate significance of Tukey's *post hoc* tests *vs.* control: *, P<0.05; **, P<0.01; ***, P<0.001. ANOVA of the effect of treatment on LDLC was significant (F = 11.173, P = 0.001). The following pairwise comparisons of individual dose groups vs. control were significant according to Tukey's post hoc tests: 0.1 mg/kg, t = -4.236, P = 0.007; 0.3 mg/kg, t = -5.448, P = 0.001.

C) Administration of ALN-PCS by a single initial dose of 1 mg/kg followed by 3 once monthly lower doses (up to 0.3 mg/kg) delivered by 60-minute IV infusion.. There were no effects of a control formulated siRNA (data not shown) in any of the parameters evaluated in the study.: Graph shows PCSK9, LDL-C and HDL-C values relative to pre-dose values (day-1 and day-3).

Phase 1 trial — pharmacokinetic data

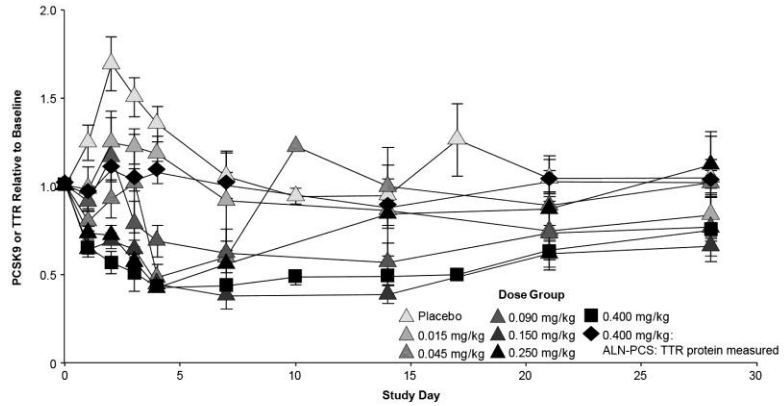


Supplemental Figure 2. PK analysis of ALN-PCS. PK analysis of lead siRNA in ALN-PCS, the siRNA exposure was observed up to 21 days for higher doses. **a)** The AUC increased with increased dose and the AUC data trend suggest that in general AUC was dose-proportional, note that one subject from the 0.15 mg/kg dose group had much greater AUC compared to the other 2 subjects.

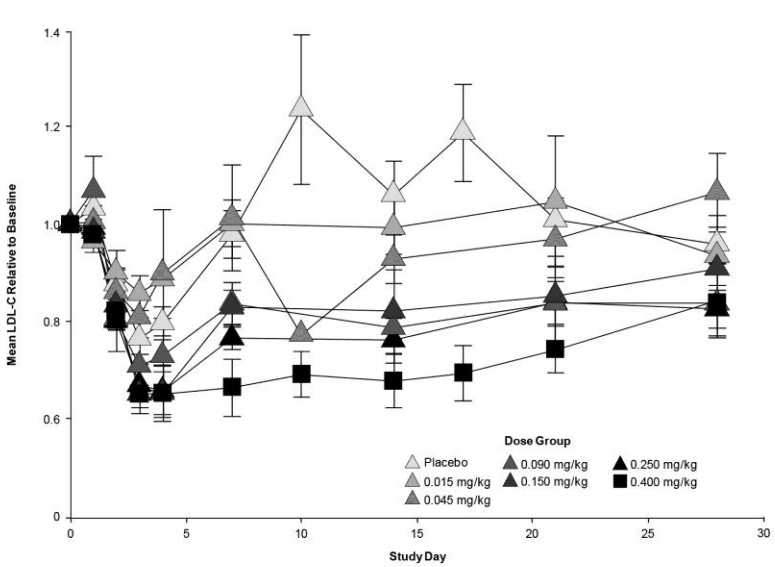
b) The Cmax increased with increased dose in linear and approximately dose-proportional manner.

Phase 1 trial— pharmacodynamic data; PCSK9 protein and LDL cholesterol concentrations relative to baseline

a



b



Supplemental Figure 3. PCSK9, TTR, and LDL-C lowering relative to baseline. a) Plasma PCSK9 (triangles) and serum TTR (diamonds) levels relative to baseline. Symbols are means ±1 SEM. Protein levels were normalized per-patient to baseline. Note: N=1 for 0.045 mg/kg group at Day 10.

b) Serum LDL-C levels relative to baseline. Symbols are means ±1 SEM. Note: N=1 for 0.045 mg/kg group at Day 10; N=2 for Placebo group at Days 10, 17.

Supplemental Methods

siRNA

siRNA sequence, selection and criteria have been previously described. [33] Briefly the siRNA is a synthetic double stranded (duplex) RNA oligonucleotide, also known as a small interfering RNA (siRNA). It is formed by the hybridization of two partially complementary single strands of RNA: the sense strand, and the antisense strand. Each strand consists of 21 ribo- or 2'-OMe nucleotides with the 3' end capped with two thymidine units. Hybridization occurs across 19 nucleotide basepairs leaving a two-thymidine overhang at each side of the duplex. All the ribo- and 2'-OMe nucleosides are connected with naturally occurring phosphodiester functional groups. The two thymidine units at the 3' end of each strand are connected with a phosphorothioate linkage. All the phosphodiester and phosphorothioate groups link the 3' and 5' oxygen of sequential nucleoside. ALN-PCS is comprised of a siRNA formulated in an LNP (AF-011) which comprises four-components: an ionizable lipid MC3, a helper lipid (DSPC), cholesterol, and PEG (PEG- DSPC). [34] The LNP is approximately 70-80 nanometers in size. The composition, size and charge of the particle allow it entry only to fenestrated tissues and effectively silence hepatocyte RNAs. [34]

Lipid nanoparticle formulation

The LNP formulation utilized for ALN-PCS has been previously described.[34]

Human and NHP PCSK9 ELISA assays

The PCSK9 sandwich ELISA to detect cynomolgous monkey PCSK9 was carried out outlined previously. [33] Alternatively the commercially available CircuLex Human PCSK9 ELISA Kit

Cat# Cy-8079 was utilized to measure cynomolgus or human PCSK9 plasma protein. The CircuLex Human PCSK9 ELISA Kit employs the quantitative sandwich enzyme immunoassay technique and was run according to manufacturer's instructions. Briefly, an antibody specific for PCSK9 was pre-coated onto a microplate. Internally produced cynomolgus PCSK9-6HIS protein standard Lot #567-163 or a control protein provided by the manufacturer was diluted with Dilution Buffer from 0.1mg/mL to 40ng/mL then 1:2 was used to create the standard curve ranging from 0.625 to 40 ng/mL. Plasma samples were diluted 1:50 with Dilution Buffer before addition to wells. 100uL diluted sample or standard was added to the plate and incubated for 1 hour at room temperature on an orbital shaker (300rpm). Plates were washed four times by filling all wells with 350uL wash buffer, then flicking out the entire volume. After washing, 100uL HRP conjugated monoclonal antibody specific for PCSK9 was added to each well and incubated for 1 hour at room temperature on an orbital shaker (300rpm). Wash step was repeated as described above. 100uL tetra-methylbenzidine Substrate Reagent was added to the plate and incubated for 30 minutes in the dark. The reaction was stopped by addition of H_2SO_4 acidic solution and absorbance of the resulting yellow product was measured at 450 nm.

NHP studies

Treatment of the animals was conducted by a certified contract research organization in accordance with the testing facility's standard operating procedure, which adheres to the regulations outlined in the United States Department of Agriculture Animal Welfare Act (9 CFR, Parts 1–3) and the conditions specified in the *Guide for the Care and Use of Laboratory Animals* (ILAR publication, 1996, National Academy Press). On the first day of dosing (day 1), all monkeys were given a single 15 min or 60 min i.v. infusion of ALN-PCS. Blood samples were collected for pharmacodynamic analysis at various time points after dose administration. Serum

chemistry experiments were carried out at Charles River Laboratories via direct measurements of LDLc, HDLc, TGs, or Tc on the Olympus AU2700.

β-quantification

A standard β-quantification method was used as described by the NIH guidelines: In brief: preparative ultracentrifugation was used to separate the various lipoprotein classes. After centrifuging at 40,000 rpm for 18 – 22 hrs at 10 ºC, the very low-density lipoprotein (VLDL) and chylomicrons were collected in the supernatant (top fraction < 1.006 density). The LDL, intermediate density lipoprotein (IDL), Lp(a), and high-density lipoprotein (HDL) were collected in the infranatant (bottom fraction > 1.006). The supernatant and infranatant fractions were separated by a tube-slicing technique and were quantitatively removed and reconstituted to a set volume (3 mL). The cholesterol concentration of the infranatant was measured using an Olympus AU2700 (Refer to Medpace Reference Laboratories (MRL, Leuven Belgium) SOP GL-CH-75-S), Total Cholesterol Measurement on Olympus AU2700). For review of method and comparison to other methods see Miida et. al., 2012. [40]

Cytokines

Cytokines were measured in serum samples obtained pre-dose and 2, 6 and 24 hours post-infusion for the first and third doses. Measurements of IFN-α, IFN-γ, IL-6, IL-12, TNF-α, IL-1β, IL-1RA, G-CSF and IP-10 were performed at Charles River Laboratories Preclinical Services PCS-MTL.

Pharmacokinetics

For pharmacokinetic analyses, plasma and plasma filtrate from specified time points were analyzed for total siRNA by QPS, LLC (Newark, Delaware). Analysis of the concentration-time

data was performed and the PK profile of each subject characterized by non-compartmental analysis of each siRNA plasma concentration using validated computer software (WinNonlin, version 3.2, Pharsight Corp., Mountain View, California, USA) at Alnylam Pharmaceuticals.